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Synergistic interaction between quercetin and doxorubicin on MCF-7 human breast cancer cell line

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ABSTRACT

The effectiveness of doxorubicin has decreased due to resistance of cancer cells. One of the natural ingredients that are proven to reduce the resistance to anticancer is quercetin. Quercetin interacts with doxorubicin via a competition of P-glycoprotein (P-gp) transporter activity. The aim of this study is to evaluate the interaction of quercetin and doxorubicin as cytotoxicity effect on MCF-7 cells. Cytotoxicity test was conducted by the MTT method. Mechanism of interaction between doxorubicin and quercetin was evaluated with isobologram analysis. Doxorubicin and quercetin inhibited the growth of MCF-7 cells significantly. Doxorubicin and quercetin respectively had IC_{50} of 21 μM and 103 μM. The interaction of doxorubicin and quercetin were characterized by the amount of doxorubicin IC_{50} equivalent and quercetin IC_{50} equivalent less than 1 and the point-intercept of each IC_{50} notation equivalent plotted on the graph below the additive line. Analysis of isobolograms indicated that the interaction doxorubisn and quercetin in each of the ratios had synergy. Quercetin can be considered to be in a combination with doxorubicin. Further study to determine other mechanisms of the interaction is required.

ABSTRAK

Penggunaan kemoterapi mencapai 98% pada penderita kanker payudara, dan 63% diantaranya menggunakan kombinasi dengan doksorubisin. Namun efektivitas pemberian doksorubisin mengalami penurunan akibat terjadinya resistensi sel kanker. Salah satu bahan alam yang terbukti dapat mengurangi resistensi antikanker adalah kuersetin. Kuersetin berinteraksi dengan doksorubisin melalui mekanisme kompetisi terhadap aktivitas transporter P-gp. Namun belum pernah dilakukan penelitian mengenai interaksi antara doksorubisin dan kuersetin. Penelitian ini bertujuan untuk mengkaji interaksi kuersetin dan doksorubisin terhadap sitotoksisitas pada sel MCF-7. Jenis penelitian ini adalah eksperimen kuasi dengan *the post test with control group design*. Aktivitas sitotoksik dilakukan dengan metode MTT. Mekanisme interaksi antara doksorubisin dan kuersetin dievaluasi dengan metode *isobologram analysis*. Doksorubisin dan kuersetin memiliki efek dalam menghambat pertumbuhan sel MCF-7 secara signifikan. Doksorubisin dan kuersetin berturut memiliki IC50 sebesar 21 μM dan 103 μM. Peningkatan atau penurunan persentase penghambatan sel oleh doksorubisin dan kuersetin bersifat *dose dependent*. Interaksi doskorubisin dan kuersetin pada uji sitotoksik sel MCF-7 bersifat sinergi minimal pada konsentrasi yang menghasilkan 50% efek maksimum, dan bersifat antagonis pada konsentrasi yang menimbulkan efek di bawah 30% efek maksimum, sedangkan interaksi yang bersifat aditif pada konsentrasi yang menimbulkan efek 40% efek maksimum. Kuersetin mempengaruhi efek sitotoksik doksorubisin pada sel MCF-7 melalui interaksi yang bersifat *dose dependent*. Interaksi ini bersifat sinergi pada konsentrasi kuersetin yang tinggi. Sebaliknya kuersetin dengan konsentrasi rendah dapat menyebabkan interaksi yang bersifat antagonis.

Key words: doxorubicin - quercetin - MCF-7 - cytotoxicity - isobologram

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INTRODUCTION

Chemotherapy still becomes the treatment of choice on various types of cancer. In patients with breast cancer, chemotherapy is used up to 98% with 63% of them used a doxorubicin combination chemotherapy.[1,2] One of the major problems related with chemotherapy in cancer is resistance against anticancer agents. It is reported that over-expression of multi drug resistance 1 gene (MDR1) encoding a transporter protein P-glycoprotein (P-gp) in various cancer cells is associated with resistance to anticancers. P-gp belongs to the ATP binding cassette (ABC) transporter family that plays a role in efflux of anticancers from cytoplasm of cancer cells.[3,4]

Various strategies are explored to reduce the resistance of cancer cells. One strategy is the use of compounds capable of inhibiting the efflux mechanism of anticancer out of cancer cells. Some drugs are proven to be able to inhibit the efflux mechanisms such as verapamil, cyclosporin, reserpine, quinidin, yohimbine, tamoxifen, quinine, amodiaquine, praziquantel and thiabendazole.[5,6] Many natural compounds such as genistein, curcumin, kaemferol and quercetin have been reported to interact with the P-gp and found to sensitize cancer cells to anticancer.[6-8]

Quercetin is a flavonoid contained in many fruits or plants such as apples, berries, grapes, garlic, tea, tomatoes, grains, nuts, ginkgo biloba, *Hipericum perforatum* (St. John's wort), and *Sambucus canadenesis*.[9,10] The interaction between quercetin and doxorubicin has been investigated by some authors, however the mechanism of the interaction remains unclear. Quercetin was reported stimulates efflux of doxorubicin by P-gp-expressing multidrug resistant cells.[11,12] Furthermore, Shapiro and Ling[13] reported that quercetin directly stimulates transport of doxorubicin that interact preferentially with the R site of P-gp-rich plasma membrane vesicles from Chinese hamster ovary CH(R)B30 cells by binding to the H site. In contrast, the recent studies showed that quercetin inhibits transport of doxorubicin from cancer cells. Hayeshi *et al.*[8] reported that quercetin inhibits P-gp mediated [^{3}H]-taxol efflux in Caco-2 cells. In addition, quercetin is reported to potentiate doxorubicin mediated antitumor effects against liver cancer and to increase doxorubicin effect in the highly invasive breast cancer cells.[14,15] This study was conducted to investigate the interaction between quercetin and doxorubicin on MCF-7 human breast cancer cell line.

MATERIALS AND METHODS

Chemical

Doxorubicin (Ebedoxo, Ebewe Pharma), quercetin (Sigma-Aldrich), dimethyl sulfoxide (DMSO) (Sigma-Aldrich), RPMI 1640 medium (Gibco), fetal bovine serum (FBS) (Gibco), amphotericin B (Gibco), L-glutamine (Sigma-Aldrich), penicillin-streptomycin (Penstrep®-Gibco), trypsin EDTA (Gibco), 4-(2-hydrocyethyl) piperazine-1-ethanesulphonic acid (HEPES) (Sigma-Aldrich), sodium bicarbonat (Nacalai Tesque), phosphate buffer saline (PBS) (Invitrogen), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Bio Basic Inc.), sodium dodesil sulfat (SDS) (Merck), acid chloride (Merck) were used in this study.

Cell culture

Human breast cancer cell lines MCF-7 were obtained from collections of Department of Parasitology, Faculty of Medicine, Universitas Gadjah Mada. Cells were cultured in culture flasks containing complete RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 μg/mL of streptomycin,

and 100 mg/mL of penicillin. Cells in culture flasks were placed in 5% CO_2 incubator at 37°C and every three days medium was replaced with complete RPMI 1640 medium. Confluent cells were trypsinized, and harvested cells were used for experiments. The study has been approved by the the Medical and Health Research Ethic Committee, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta.

Drug solution preparation

Stock solutions of the tested drugs were prepared at 1000 µM in DMSO, which, once diluted, had no effect on cell growth at final concentration of 0.05%. On day of the experiment, dilutions were prepared from stock solution with culture medium. Concentration-cell growth assays were first conducted to obatin the inhibitory concentration of 50% (IC_{50}) of individual drugs, doxorubicin and quercetin. For the combination assay, drug dilutions were made to allow the IC_{50} of the individual drugs to fall at three or four serial dilution as follow. The stock solutions containing 591.24; 259.62; and 147,81µM quercetin were combined with 17.24 µM doxorubicin, representing approximate quercetin-to-doxorubicin IC_{50}-equivalent ratios of 1:34; 1:17; and 1:9. Whereas the stock solutions containing 73.91; 36.95; 18.48; and 9.24 µM quercetin were combined with 43.1 µM doxorubicin IC_{50}-equivalent ratios of 1:2; 1:0.8; 1:0.4; and 1:0.2. The cells were treated with serial dilutions (2- to 8-fold diluted) of the stock solutions. Controls were processed similarly but without drugs.

Cytotoxicity assay

Cytotoxicity of doxorubicin or quercetin or its combination in various concentration was evaluated on MCF-7 cells using the MTT assay as developed by Mosmann after modification.[16] One hundred mL of cell cultures were distributed in triplicate in 96-wells microplates at a density of 1 x 10^4 cells per well and then 100 mL of complete RPMI 1640 medium were added. The cell cultures were then placed in 5% CO_2 incubator at 37°C for 24 hours. After incubation, the medium was removed and replaced with new complete RPMI 1640 medium containing various concentrations of doxorubicin or quercetin or its combination. The cell cultures containing tested drugs were then incubated again in 5% CO_2 incubator at 37°C for 24 hours. Following incubation, the medium was removed and the cell cultures were resuspended in RPMI 1640 medium, 10 mL of 5 mg/mL MTT [3-9,4,5-dimethylthiazole-2-yl-2,5-diphenyltetrazolium bromide] and then further incubated for 4 hours. The reaction was stopped by adding 100 mL of 10% sodium dodecyl sulfate (SDS) in 0.01N HCl. The microculture plates were then shaken gently for 5 minutes, covered with aluminium foil and incubated at room temperature for 24 hours. Absorbance of the microculture plates was measured in an ELISA plate reader at l_{max} 595 nm. The absorbance values were directly proportional to the number of live cells. The absorbance values in the presence of tested drugs were compared with that of control cultures without tested drugs to obtain cells growth inhibition. The IC_{50} values were then determined by probit analysis based on the relationship between log concentrations versus the percentage of cells growth inhibition.

Doxorubicin and quercetin interaction evaluation

Doxorubicin and quercetin interaction was evaluated using the fixed ratio method, where the doxorubicin and quercetin concentrations were present in concentrations fixed ratio corresponding to the IC_{50} equivalents concentration of single drug. The IC_{50} equivalents concentration were caculated by the equation below.[17]

$$IC_{50} \text{ equivalent concentration} = \frac{C_{D,50}}{IC_{50,D}} + \frac{C_{Q,50}}{IC_{50,Q}}$$

$C_{D,50}$ and $C_{Q,50}$ were consecutively concentration of doxorubicin and in a combination that generates 50% of the maximal combination effect. $IC_{50,D}$ $IC_{50,Q}$ were consecutively single-concentration of doxorubicin and quercetin that generated 50% of the maximal single effect.

Following IC_{50} equivalent concentration was calculated for each point, isobolograms were plotted. Interaction of two drugs was considered as additive if the IC_{50} equivalent of two drugs was parallel to the diagonal line. Synergism or antagonism was considered to exist between the two drugs if the IC_{50} equivalent of the combined drugs was lower or higher than this line, respectively.

TABLE 1. IC_{50} values (mean ± SD in μM) of doxorubicin and quercetin in each of combinations on MCF-7 cell line

Ratio combination D:Q	Doxorubicin	Quercetin
1:00	21.47 ± 1.81	-
1:34	2.31 ± 0.06	79.33 ± 2.08
1:17	3.74 ± 0.07	64.04 ± 1.20
1:90	5.00 ± 0.33	42.87 ± 2.85
1:20	11.35 ± 0.65	19.45 ± 1.12
1:0.8	12.95 ± 0.35	11.20 ± 0.18
1:0.4	13.75 ± 0.41	5.90 ± 0.18
1:0.2	13.42 ± 0.42	2.88 ± 0.09
0:1.0	-	103.12 ± 5.23

Note : D= doxorubicin; Q= quercetin; SD= standard deviation

TABLE 2. IC_{50} equivalent (mean ± SD) in each of combinations on MCF-7 cell line

Ratio D:Q	IC_{50} equivalent concentration		Interaction
	Doxorubicin	Quercetin	
1:0	1.00±0.00	0	-
1:34	0.11±0.01	0.77±0.02	Synergy
1:17	0.18±0.02	0.62±0.02	Synergy
1:9	0.24±0.03	0.42±0.01	Synergy
1:2	0.53±0.08	0.19±0.01	Synergy
1:0.8	0.61±0.07	0.11±0.00	Synergy
1:0.4	0.64±0.04	0.06±0.01	Synergy
1:0.2	0.63±0.07	0.03±0.00	Synergy
0:1	0	1.00±0.00	-

D: doxorubicin; Q: quercetin; SD= standard deviation

RESULTS

TABLE 1 summarizes the IC_{50} values of each of the seven ratios combinations in between doxorubicin and quercetin on MCF-7 cell lines. The IC_{50} value of doxorubicin was 21.47 μM, whereas the IC_{50} value of quercetin was 103.12 μM. It was indicated that doxorubicin had more cytotoxic effect on MCF-7 cell than quercetin.

TABLE 2 summarizes the IC_{50} equivalent values of each of the seven ratios combinations in between doxorubicin and quercetin on MCF-7 cell lines. A synergisticinteraction was observed between doxorubicin and quercetion in all the combination ratio as expressed by the sum of IC_{50} equivalent values of doxorubicin with quercetin of ≤ 1. Isobologram analysis (FIGURE 1) supported this results. The IC_{50} equivalent of the combination doxorubicin and quercetin was lower than the diagonal line.

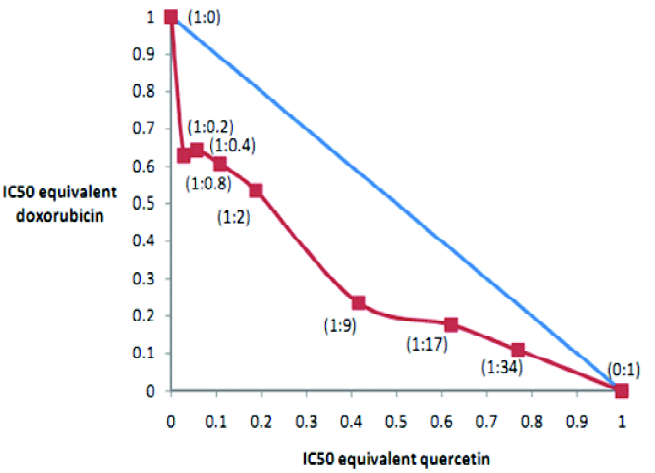

FIGURE 1. Isobologram analysis of the combination doxorubicin and quercetin on MCF-7 cell lines

DISCUSSION

Recent studies suggest that quercetin can enhance the response of tumors to chemotherapy. However, the mechanism by which quercetin enhances the sensitivity of tumor cells to anticancer drugs remains elusive. Several studies focus on the effect of quercetin on the modulation of P-gp activity on cancer cells has been conducted by some authors with different results. One group of authors reported that quercetin stimulates efflux of anticancer drugs by P-gp of multidrug-resistant cells.[11-13,18] In contrast, another groups reported that quercetin inhibites efflux of anticancer drugs.[8,14,15]

Our study clearly demonstrated a synergic cytotoxic effect of quercetin and doxorubicin on MCF-7 breast cancer cell line at all combination ratios. The synergistic effect was characterized by the sum of doxorubicin IC_{50} equivalent and quercetin IC_{50} equivalent less than one. This synergistic was also supported by isobologram analysis which the IC_{50} equivalent of the combination doxorubicin and quercetin was lower than the diagonal line. Furthermore, the combination of doxorubicin and quercetin with concentration ratio of 1:0.2 had the best synergisticcytotoxic effect compared to the other combinations.

The synergic effect of quercetin and doxorubicin has been reported in the previous studies. Staedler *et al.*[19] demonstrated that quercetin potentiates antitumor effects of doxorubicin specifically in the highly invasive breast cancer cells and attenuated unwanted cytotoxicity to non-tumoral cells. Moreover, Du *et al.*[20] reported that dietary quercetin combining intratumoral doxorubicin injection synergistically induces potent rejection of 4T1 breast cancer and leads to long-term, tumor-free survival in mice bearing established breast tumor, whereas quercetin or doxorubicin alone fails to cure tumor-bearing mice. Du *et al.*[21] also reported that quercetin suppress tumor growth and prolongs survival in BALB/c mice bearing 4T1 breast cancer. Importantly, the quercetin enhances therapeutic efficacy of doxorubicin and simultaneously reduced doxorubicin-induced side effects.

The mechanism of synergistic interaction between quercetin and doxorubicin on human cancer cells has been also investigated by some authors. Quercetin was proven to inhibit to the

ATP-binding site of P-gp transporter responsible for the efflux of doxorubicin leads to sensitivity to doxorubicin in MDR positive MCF-7 cells.[8,22] In addition, *in vivo* study in mice cancer showed that quercetin selectively sensitized doxorubicin-induced cytotoxicity against liver cancer cells. Quercetin increased doxorubicin-mediated apoptosis in hepatoma cells by induction p53 and downregulation Bcl-xl expressions.[14]

The another possibility mechanism of synergistic interation between quercetin and doxorubicin has been postulated. Quercetin increased the bioavailability of oral doxorubicin by enhancement doxorubicin absorption in the gastrointestinal tract through quercetin-induced inhibition of P-gp and reduction first-pass metabolism of doxorubicin through quercetin-induced inhibition of CYP3A in the small intestine and/or in the liver.[23] Furthermore, quercetin has been reported to modulate immune system in mice by induction lymphocyte proliferation and regulation Th1/Th2 cytokine imbalance. Combination of quercetin and intratumoral doxorubicin injection further induced a persistent T-cell tumor-specific responses.[20]

CONCLUSION

In conclusion, quercetin can increase the sensitivity of human breast cancer cell line MCF-7 to doxorubicin through synergistic interaction. The combination of quercetin with doxorubicin may represent a novel strategy for increasing efficacy and reducing side effect of doxorubicin. Clinical study to evaluate the efficacy of the combination is needed.

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REFERENCES

1. Hancke K, Denkinger MD, Konig J, Kurzeder, C., Wöckel A, Herr D, *et al*. Standard treatment of female patients with breast cancer decreases substantially for women aged 70 years and older: a German clinical cohort study. Ann Oncol 2010; 21(4): 748-53.
2. Sautter-Bihl ML, Sauchon R, Gerber B. Adjuvant therapy for women over age 65 with breast cancer. Dtsch Arztebl Int 2011; 108(21): 365-71.
3. van Brussel JP, van Steenbrugge GJ, Romijn JC, Schröder FH, Mickisch GH. Chemosensitivity of prostate cancer cell lines and expression of multidrug resistance-related proteins. Eur J Cancer 1999; 35(4):664-71.
4. Pasquier J, Magal P, Lecomte CB, Webb G, Foll FL. Consequences of cell-to-cell P-glycoprotein transfer on acquired multidrug resistance in breast cancer: a cell population dynamics model. Biol Direct 2011; 6(5): 1-16.
5. Dantzig AH, de Alwis DP, Burgess M. Considerations in the design and development of transport inhibitors as adjuncts to drug therapy. Adv Drug Deliv Rev 2003; 55(1): 133-50.
6. Wortelboer HM, Usta M, van der Velde AE, Boersma MG, Spenkelink B, van Zanden JJ, *et al*. Interplay between MRP inhibition and metabolism of MRP inhibitors: the case of curcumin. Chem Res Toxicol 2003; 16(12):1642-51.
7. Limtrakul P, Khantamat O, Pintha K. Inhibition of P-glycoprotein function and expression by kaempferol and quercetin. J Chemother 2005; 17(1):86-95.
8. Hayeshi R, Masimirembwa C, Mukanganyama S, Ungell AL. The potential inhibitory effect of antiparasitic drugs and natural products on P-glycoprotein mediated efflux. Eur J Pharm Sci 2006; 29(1):70-81.
9. Williamson G, Manach C. Bioavailability and bioefficacy of polyphenols in humans II: Review of 93 intervention studies. Am J Clin l Nutr 2005; 81 (Suppl 1): S243-55.
10. Anonim. USDA Database for the flavonoid content of selected foods. Baltimore: U.S. Department of Agriculture, 2003.
11. Phang JM, Poore CM, Lopaczynska J, & Yeh GC Flavonol stimulated efflux of 7,12 dimethyl-benz(a)anthracene in multidrugresistant breast cancer cells, Cancer Res 1993; 53(24): 5977-81.

12. Critchfield JW, Welsh CJ, Phang JM. & Yeh GC. Modulation of adriamycin accumulation and efflux by flavonoids in HCT-15 colon cells: activation of P-glycoprotein as a possible mechanism. Biochem Pharmacol 1994; 48(7): 1437-45.
13. Shapiro AB, Ling V. Positively cooperative sites for drug transport by P-glycoprotein with distinct drug specificities. Eur J Biochem 1997; 250(6): 130-7.
14. Wang G, Zhang J, Liu L, Sharma S, Dong Q. Quercetin potentiates doxorubicin mediated antitumor effects against liver cancer through p53/ Bcl-xl. PloS One 2012; 7(12): e51764. Doi: 10.1371/journal.pone.0051764
15. Staedler D, Idrizi E, Kenzaoui BH, Juillerat-Jeanneret L. Drug combinations with quercetin: doxorubicin plus quercetin in human breast cancer cells. Cancer Chemother Pharmacol 2011; 68(5):1161-72.
16. Mossman T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65(1-2):55-63.
17. Zhao L, Au JLS, Wientjes MG. Comparison of methods for evaluating drug-drug interaction. Front Biosci (Elite ed.) 2010; 2: 241-9.
18. Václavíková R, Kondrová E, Ehrlichová M, Boumendjel A, Kovár J, Stopka P, *et al.* The effect of flavonoid derivatives on doxorubicin transport and metabolism. Bioorg Med Chem 2008; 16(4): 2034-42.
19. Staedler D, Idrizi E, Kenzaoui BH, Juillerat-Jeanneret L. Drug combinations with quercetin: doxorubicin plus quercetin in human breast cancer cells. Cancer Chemother Pharmacol 2011; 68(5): 1162-72.
20. Du G, Lin H, Yang Y, Zhang S, Wu X, Wang M, *et al.* Dietary quercetin combining intratumoral doxorubicin injection synergistically induces rejection of established breast cancer in mice. Int Immunopharmacol 2010; 10(7):819-26.
21. Du G, Lin H, Wang M, Zhang S, Wu X, Lu L, *et al.* Quercetin greatly improved therapeutic index of doxorubicin against 4T1 breast cancer by its opposing effects on HIF-1α in tumor and normal cells. Cancer Chemother Pharmacol 2010; 65(2):277-87.
22. Scambia G, Ranelletti F, Panici PB, Vincenzo R, Bonanno G, Ferrandina G, *et al.* Quercetin potentiates the effect of adriamycin in a multi-drug-resistant MCF-7 human breast cancer cell line: P-glycoprotein as a possible target. Cancer Chemother Pharmacol 1994; 34(6):459-64.
23. Choi JS, Piao YJ, Kang KW. Effects of quercetin on the bioavailability of doxorubicin in rats: role of CYP3A4 and P-gp inhibition by quercetin. Arch Pharm Res 2011; 34(4):607-13.